Effects of H^+-ATPase Activities on Cd^{2+} Movement Induced by NADH-linked Electron Transfer in Root Plasma Membrane Vesicles

Shu-I Tu, Deidre Patterson, David Brauer, and An-Fei Hsu

Summary

The vanadate-sensitive H^+-ATPase activities, proton pumping and ATP hydrolysis of the corn root plasma membrane vesicles were not significantly affected by the electron transfer from NADH to ferricyanide. Similarly, the electron transfer activity was also not affected by activation of the H^+-ATPase. These results suggested a lack of direct interaction between the two energy-yielding processes in the plasma membrane. The NADH-linked electron transfer induced a Cd^{2+} concentration-dependent increase in the light scattering of the membrane vesicles. The increase was previously determined to be an indication of Cd^{2+} uptake by the vesicles (Tu et al., 1996). The rate of the light scattering increase was significantly decreased when the ATPase was activated by the addition of Mg-ATP. Neither free ATP nor free Mg^{2+} had the inhibitory effect on the light scattering change. The inhibition of the light scattering increase associated with the activation of the ATPase exhibited a pH dependence and vanadate sensitivity similar to those of the ATPase activities. The results suggest that the ATPase activities could regulate the Cd^{2+} movement activated by the NADH-linked electron transfer.

Key words: Corn (Zea mays), cadmium transport, NADH-linked electron transfer, root plasma membrane, vanadate-sensitive H^+-ATPase.

Introduction

The plasma membrane (PM) of plant root cells exhibits activities of both vanadate-sensitive H^+-ATPase (Hodges and Leonard, 1974) and NADH-linked electron transfer (Lin, 1982). The ATPase contains a 100-kDa peptide chain with up to 10 α-helical membrane segments (Wach et al., 1992) and forms a phosphorylated intermediate (Briskin and Leonard, 1982) during the hydrolysis of Mg-ATP. The ATPase, like many other membrane bound ATPases, converts part of the hydrolysis energy to a transmembrane proton electrochemical potential ($\Delta\mu_{H+}$), which contains a pH gradient (ΔpH) and a membrane potential ($\Delta\psi$). The possible physiological roles of the ATPase in nutrient uptake have been proposed (Serrano, 1989) to follow the general chemiosmotic concept (Racker, 1979).

In contrast, the properties of NADH-linked electron transfer are less well characterized. The NADH-supported electron transfer system contains of NADH-cytochrome *c* reductase (Larsson, 1985), flavin-NADH dehydrogenase (Ramirez et al., 1984) activities and a *b*-type cytochrome (Leong et al., 1981). Particles enriched with multiple NADH-linked redox activities have been obtained from corn root plasma membrane vesicles by detergent treatments (Luster and Buckout, 1988). It was previously determined (Askerlund et al., 1988) that the NADH oxidation and ferricyanide reduction occured mainly on the cytoplasmic face of the plasma membrane. Kinetic analysis on the reduction of cytochrome *c* and ferricyanide by NADH (Tu et al., 1993), suggest that the reduction of these two artificial substrates should occur at

[1] Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

BENSADOUM, A. and D. WEINSTEIN: Assay of proteins in the presence of interfering materials. Anal. Biochem. *70,* 241–250 (1976).

BRAUER, D., A-F. HSU, and S-I. TU: Factors associated with the instability of nitrate-insensitive proton transport by maize root microsomes. Plant Physiol. *87,* 598–602 (1988).

BRISKIN, D. P. and R. T. LEONARD: Partial characterization of a phosphorylated intermediate associated with the plasma membrane ATPase of corn roots. Proc. Natl. Acad. Sci. USA *79,* 6922–6926 (1982).

CRANE, F. L., H. LOW, and M. G. CLARK: Plasma membrane redox enzymes. In: MARTONOSI, A. N. (ed.): The Enzymes of Biological Membranes, pp. 465–510. Plenum Press, New York (1985).

KLOBUS, G. and J. BUCZEK: The role of plasma membrane oxidoreductase activity in proton transport. J. Plant Physiol. *146,* 103–107 (1995).

HODGES, T. K. and R. T. LEONARD: Purification of a plasma membrane-bound adenosine triphosphatase from roots. Methods Enzymol. *32,* 392–406 (1974).

HSU, A-F., D. BRAUER, and S-I. TU: Characterization of reconstituted plasma membrane H^+-ATPase from maize roots. Physiol. Plant. *76,* 544–550 (1989).

LARSSON, C.: Plasma membranes. In: LINEKENS, H. F. and J. F. JACKSON (eds.): Methods of Plant Analysis (NS), pp. 85–104. Springer-Verlag, Berlin (1985).

LEONG, T. Y., R. D. VIERSTRA, and W. R. BRIGGS: A blue light-sensitive cytochrome-flavin complex from corn coleoptiles. Further characterization. Photochem. Photobiol. *34,* 697–703 (1981).

LIN, W.: Responses of corn root protoplasts to exogenous reduced nicotinamide adenine dinucleotide: oxygen consumption, ion uptake, and membrane potential. Proc. Natl. Acad. Sci. USA *79,* 3773–3776 (1982).

LUSTER, D. G. and T. J. BUCKHOUT: Characterization and partial purification of multiple electron transport activities in plasma membrane from maize (*Zea mays*) roots. Physiol. Plant. *73,* 339–347 (1988).

MITCHELL, P.: Vectorial chemistry and the molecular mechanism of chemiosmotic coupling: power transmission by protoicity. FEBS Lett. *59,* 137–139 (1975).

MØLLER, I. M. and F. L. CRANE: Redox process in plant plasma membrane. In: LARSSON, C. and M. MØLLER (eds.): The Plant Plasma Membrane: Structure, Function, and Molecular Biology. pp. 93–126. Springer, Berlin (1990).

RACKER, E.: Transport of ions. Account Chem. Res. *12,* 338–344 (1979).

RAMIREZ, J. M., G. G. GALLEGO, and R. SERRANO: Electron transfer constituents in plasma membrane fractions of *Avena sativa* and *Saccharomyces cerevisiae.* Plant Sci. Lett. *34,* 103–110 (1984).

RUBINSTEIN, B. and D. G. LUSTER: Plasma membrane redox activity: Components and Role in plant processes. Annu. Rev. Plant Physiol. Plant Mol. Biol. *44,* 131–155 (1993).

SERRANO, R.: Structure and function of plasma membrane ATPase. Ann. Rev. Plant Physiol. Plant Mol. Biol. *40,* 61–94 (1989).

TAQUI KHAN, M. M. and A. E. MARTELL: Metal chelates of adenosine diphosphoric and monophosphoric acids. J. Amer. Chem. Soc. *84,* 3037–3041 (1966).

THIELMANN, J., N. E. TOLBERT, A. GOYAL, and H. SENGER: Two systems for concentrating CO_2 and bicarbonate during photosynthesis by *Scendesmus.* Plant Physiol. *92,* 622–629 (1990).

TU, S-I., J. N. BROUILLETTE, and G. NAGAHASHI: Proton pumping kinetics and origin of nitrate inhibition of tonoplast-type H^+-ATPase. Arch. Biochem. Biophys. *266,* 289–297 (1987).

TU, S-I., D. PATTERSON, and D. BRAUER: NADH-linked ferricyanide and cytochrome *c* reduction activities in corn root plasma membrane. Plant Cell Physiol. *34,* 1213–1218 (1993).

– – – Differential inhibition of corn vanadate-sensitive H^+-ATPase activities by fluorescamine and its derivatives. Plant Physiol. Biochem. *32,* 93–104 (1994).

TU, S-I., D. PATTERSON, S. SHEN, D. BRAUER, and A. F. HSU: NADH-linked electron transfer induces Cd^{2+} movement in corn root plasma membrane vesicles. Plant Cell Physiol. *37,* 141–146 (1996).

WACH, A., A. SCHLESSER, and A. GOFFEAU: An alignment of 17 deduced protein sequences from plant, fungi, and protozoa H^+-ATPase genes. J Bioenerg. Biomembr. *24,* 309–317 (1992).

Table 4: Effects of pH on the Kinetics ($t_{1/2}$) of the Light Scattering Increase[1].

pH	$t_{1/2}$, s (without Mg-ATP) ± 5 %	$t_{1/2}$, s (with Mg-ATP), ± 5 %	Relative change in $t_{1/2}$[2]
6.0	34	134	3.9
6.5	24	109	4.5
7.0	23	82	3.6
7.5	23	67	2.9

[1] The time course of the light scattering increase of the plasma membrane vesicles in solutions of different pH and with/without ATP were obtained as described in Fig. 1. The time needed to reach one half of the maximum increse was measured. The data shown represented the average of two independent measurements with errors ± 5 %.

[2] The relative change is defined as the ratio of $t_{1/2}$ in the presence of ATP to that in the absence of ATP.

its inhibitory effect to the light scattering change. They also showed that the final maximum increase of light scattering was not significantly affected by the addition of ATP at a given pH. A qualitative indication on the pH effect was summarized in Table 4. The time needed to reach one-half of the maximum increase ($t_{½}$) at a given pH was determined. Between pH 6.5 and 7.5 (near constant electron transfer activity), the change in $t_{½}$ of the light scattering time course decreased as the ATPase activity decreased. At pH 6.0, both the electron transfer and ATPase activities decreased. Thus, the $t_{½}$ of the light scattering change exhibited further decreases. The results suggested that the slow down in the Cd^{2+}-movement powered by the NADH-linked electron transfer appeared to be quantitatively related to the ATPase activity.

Possible origins of observed H^+-ATPase effects

The H^+-ATPase catalyzes the hydrolysis of ATP and activates an energetic proton movement across the membrane. The ion movement generates a proton electrochemical potential with a high acidity and a positive electric potential in the interior aqueous space of the inside-out plasma membrane vesicles. This thermodynamic condition may serve as a barrier for moving Cd^{2+} ions in the same direction of proton pumping (uptake by the vesicles). However, if the uptake of Cd^{2+} is linked to the return of pumped protons (H^+/Cd^{2+} antiport), then the uptake should be enhanced by the transmembrane proton gradient. This prediction contradicts the observed effects of pre-incubating the plasma membrane vesicles with ATP prior to an activation of the Cd^{2+} uptake. Thus, the plasma membrane does not appear to contain a H^+/Cd^{2+} antiport mechanism for extruding Cd^{2+} from cell cytoplasm. To assess the involvement of the membrane proton electrochemical potential in the Cd^{2+}-movement, we have tested the effects of nigericin (Trace E, Fig. 1), a protonophore, and valinomycin (Trace F, Fig. 1), a K^+ ionophore. It is well established that nigericin and valinomycin can decrease the pH gradient and the membrane potential, respectively. As shown, the H^+-ATPase induced inhibition to the Cd^{2+} movement was indeed partially but significantly relieved by the presence of nigericin or valinomycin.

Discussion

It is generally accepted that the plasma membrane of plant root cells contains all essential components for nutrient uptake into the cytoplasm. The H^+-ATPase is known to provide the energy for this uptake (Racker, 1979). In contrast, the possible roles of NADH-linked electron transfer in membrane transport remain to be firmly established (Rubinstein and Luster, 1993). In our previous report (Tu et al., 1996), we demonstrated that the electron transfer induced light scattering increase of the plasma membrane vesicles in Cd^{2+}-containing media. Using phase contrast microscopy, the increase in light scattering was related to an expansion of the vesicles. Accompanying the volume increase, a decrease of Cd^{2+} concentration in the external space was observed by the use of Arsenazo III, a metallochromophore specific for divalent cations. The observed changes in light scattering and Cd^{2+} concentration were sensitive to A23187. Thus, the NADH-linked electron transfer may induce a Cd^{2+} uptake by the plasma membrane vesicles. In present study, we demonstrated that this Cd^{2+} uptake was not affected by the presence of Mg^{2+}. This result suggested that the plasma membrane might contain a very specific mechanism for the Cd^{2+} movement. Since the functional (redox-active and ATPase active) vesicles presumably assumed inside-out membrane orientation, the data suggested that NADH-linked electron transfer could activate a very specific Cd^{2+} extrusion mechanism in intact root cells.

The results described in this study seems to suggest that the observed redox-linked Cd^{2+} uptake may be regulated by a number of ATP-related activities. We noted that free ATP, presumably due to its ability to interact with some yet unidentified membrane sites, caused a vanadate-insensitive but less pronounced modification of the uptake process. The Cd^{2+} uptake was significantly inhibited by an activation of the vanadate-sensitive H^+-ATPase and an arrest of the ATPase by vanadate relieved the inhibition (Fig. 1). The activation of the H^+-ATPase should initiate ATP hydrolysis and transmembrane proton pumping. In addition, the activation might also induce certain conformational rearrangements of membrane components (Tu et al., 1994). As noted from the effects of nigericin and valinomycin, the inhibition of the Cd^{2+} movement may in part, due to the ATPase-linked transmembrane proton pumping. These results and the fact that a pronounced relief of the inhibition was observed only with the ATPase arrested by vanadate, suggest that other ATPase-related activities, e.g. conformational interactions with membrane components may also be involved in regulating the Cd^{2+} movement. However, the exact mechanism of the ATPase modulation on the electron transfer-activated Cd^{2+} transport remains to be established.

References

Askerlund, P., C. Larsson, and S. Widell: Localization of donor and acceptor sites of NADH dehydrogenase activities using inside-out and right-side-out plasma membrane vesicles from plants. FEBS Lett. *239*, 23–28 (1988).

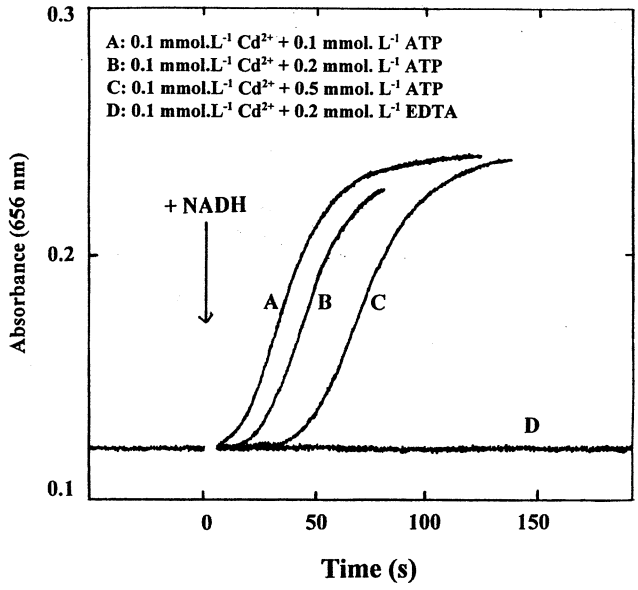

Fig. 3: Effects of Varying the Ratio of Cd/ATP to the Light Scattering Change. The light scattering change of the plasma membrane vesicles (95 µg) was measured as described in Fig. 1 in the same medium except the omission of Mg^{2+}. The concentration of added Cd^{2+} was kept as 0.1 mmol · L^{-1} but the ATP concentration increased from 0.1 mmol · L^{-1} (trace A), 0.2 mmol · L^{-1} (trace B), to 0.5 mmol · L^{-1} (trace C). The addition of 0.2 mmol · L^{-1} vanadate did not significantly alter traces A, B and C. The omission of ATP from the medium yielded a trace almost identical to A. In trace D, the ATP was replaced by 0.2 mmol · L^{-1} EDTA.

contribute, but not significantly, to the observed inhibition noted in trace B of Fig. 1.

The experiments shown in Fig. 3 also indicated that the conversion of free Cd^{2+} to Cd-ATP complex did not significantly affect the rate of scattering change. These results appeared to be contradictory to the dependence of the light scattering change on the concentration of free Cd^{2+} mentioned in Fig. 2. However, when 0.2 mmol · L^{-1} ATP was replaced by 0.2 mmol · L^{-1} EDTA, a much stronger chelator for Cd^{2+}, no redox-linked light scattering increase of the membrane vesicles was detected (trace D, Fig. 3). The results of Fig. 2 and 3 led us to conclude that the molecular species responsible for Cd^{2+} uptake in the plasma membrane vesicles should have a stronger Cd^{2+} affinity than that associated with ATP but weaker than that associated with EDTA. Thus, under the conditions of Fig. 3, the availability of free Cd^{2+} to the uptake mechanism was not affected by the weaker complex of Cd-ATP.

Effects of modulating the ATPase activity on the light scattering change

The results discussed so far supported the notion that the activation of the H^+-ATPase could slow down the Cd^{2+} movement induced by the electron transfer. To support this prediction, we measured the effects by inhibiting the ATPase activity with a specific inhibitor, vanadate. With the ATPase inhibited by vanadate, the time course of the light scattering change returned to a state (trace C, Fig. 1) similar to that obtained without ATP (trace A, Fig. 1). This result indicated a strong link between the ATPase activity and the Cd^{2+}-related light scattering change of the vesicles. It also ruled out the possibility of lowering the free Cd^{2+} concentration by Mg-ATP discussed above since the presence of vanadate should not have any effect on ATP complexation with Mg^{2+} or Cd^{2+}. While the vanadate effect provided a strong link to the H^+-ATPase, the results did not rule out the possible interactions between Cd^{2+} transport mechanism and an unidentified vanadate-sensitive process in plant systems. For example, it was demonstrated that the transport of CO_2 and bicarbonate in unicellular green algae could be inhibited by vanadate (Thielmann et al., 1990). To further test this possibility, we examined the inhibitory efficiency of the ATPase in media with different pH values. The electron transfer exhibited maximal activity over the pH range of 6.5 to 7.5 (Tu et al., 1993), but the pH profile of the ATPase showed a sharp peak at pH 6.5 (Hsu et al., 1989). A decrease in the activity of the ATPase by changing the pH of the medium from 6.5 to 7.5, considerably relieved the inhibitory effects on the time course of light scattering change (Fig. 4). Thus, the results of Fig. 4 suggested that a decrease in the ATPase activity also decreased

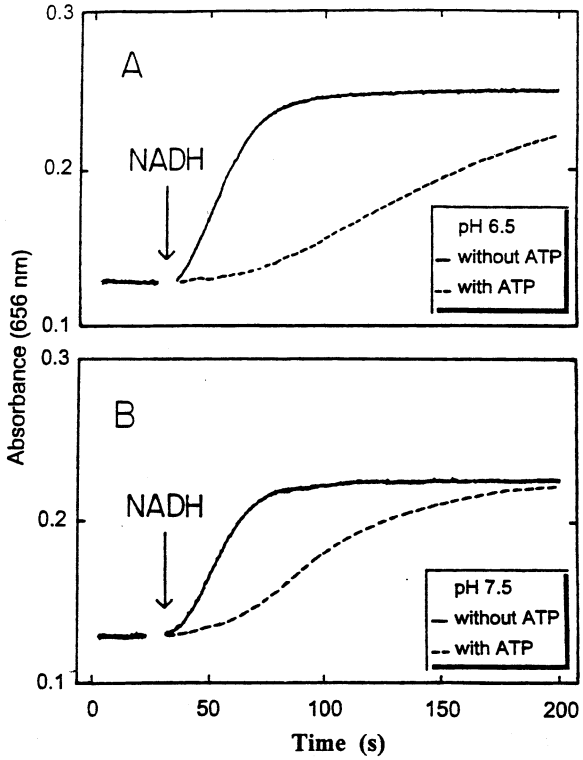

Fig. 4: Effects of pH on the Influence of ATPase on the Light Scattering Increase. The light scattering increase of the plasma membrane vesicles was monitored as described in Fig. 1. The time courses of the light scattering change in media of pH 6.5 and 7.5 are shown in Panels (A) and (B), respectively. The light scattering changes in media with and without ATP are shown as dashed and solid line traces, respectively.

the plasma membrane vesicles and does not interfere with the uptake of Cd^{2+}.

The inclusion of ATP to activate the H^+-ATPase, prior to the initiation of the electron transfer by NADH, significantly decreased the rate but not the extent of light scattering change (trace B of Fig. 1 and Fig. 4). This result suggested that the activation of the ATPase could slow down the electron transfer-induced Cd^{2+} movement. A trivial explanation for the observed increase would be a displacement of Mg^{2+} by Cd^{2+} from ATP-complexes and thus a decrease in the concentration of free Cd^{2+}. Indeed, both the extent and the rate of the light scattering increase were related to the concentration of free Cd^{2+} in the assay medium. As shown in Fig. 2, under the experimental conditions, the rate increased rapidly as the concentration increased and reached a plateau value at about 40 $\mu mol \cdot L^{-1}$. In contrast, the extent of the increase did not appear to level off in the tested concentration range of Cd^{2+}. At neutral pH, the responses of the phosphorus nmr spectrum of 10 $mmol \cdot L^{-1}$ ATP (chemical shifts and peak intensities) to the presence of 1 to 10 $mmol \cdot L^{-1}$ Cd^{2+} were almost identical to those caused by Mg^{2+} in the same concentration range (data not shown). The result indicated that Cd-ATP has a stability comparable to that of Mg-ATP. The dissociation constant of Mg-ATP in neutral pH at 25 °C, was determined as ~60.2 $\mu mol \cdot L^{-1}$ (Taqui Kahn and Martell, 1966). The experiment of trace B in Fig. 1 was conducted under the condition of excess of Mg^{2+} (2.5 $mmol \cdot L^{-1}$) to Cd^{2+} (0.1 $mmol \cdot L^{-1}$), and an ATP concentration as 1 $mmol \cdot L^{-1}$. Thus, the concentration of free Cd^{2+} only changed from 0.1 $mmol \cdot L^{-1}$ in trace A to about 60 $\mu mol \cdot L^{-1}$ in trace B of Fig. 1. As shown in Fig. 2, this decrease in free Cd^{2+} concentration should not significantly alter the rate of the light scattering change. Furthermore, no change in the extent of light scattering increase was noted in comparing traces A and B. Thus, the effects shown in trace B could not be due to the competition between Mg^{2+} and Cd^{2+} for ATP.

Although favorable for complex formation with divalent cations, under the experimental conditions of Fig. 1, a change from trace A to trace B would also introduce free ATP to the system. Thus, the observed slow down in the change of light scattering could also be due to the presence of free ATP. From the dissociation constant of Mg-ATP, the concentration of free ATP in trace B was calculated as ~0.05 $mmol \cdot L^{-1}$. By omitting Mg^{2+} from the medium to stop the activation of the H^+-ATPase but maintaining the Cd^{2+} concentration as 0.1 $mmol \cdot L^{-1}$, we noted only a slight slow down of the light scattering change with added total ATP concentration varied from 0.1 to 0.5 $mmol \cdot L^{-1}$ (Fig. 3). As the concentration of ATP increased, the lag time between addition of NADH and scattering change also increased. However, it should be emphasized that the observed effects of free ATP were not sensitive to the presence of 0.2 $mmol \cdot L^{-1}$ vanadate (data not shown). The results of Fig. 3 suggested that free ATP could

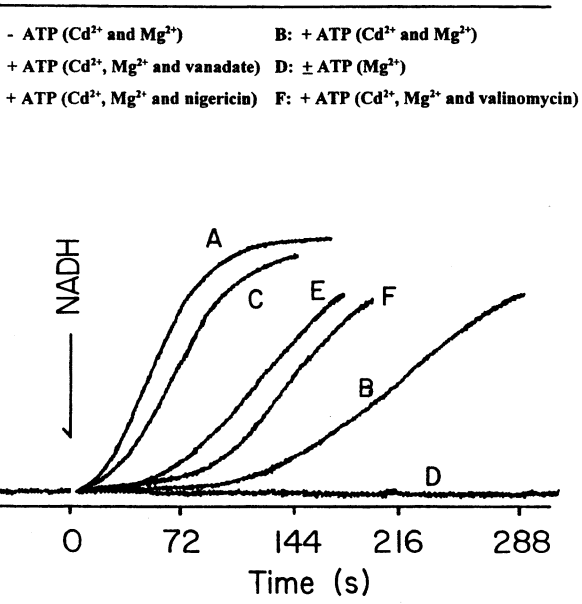

Fig. 1: Effects of Activating ATPase Upon the Light Scattering Increase Associated with NADH-linked Electron Transfer in Cd^{2+}-Containing Media. The light scattering changes of the plasma membrane vesicles (95 μg) in media with different contents were measured as described in Material and Methods. To test the effects of ATPase activity, the membrane vesicles were first incubated with the assay medium including 2.5 $mmol \cdot L^{-1}$ of $MgCl_2$ for 10 min at 22 °C before the addition of 1.0 $mmol \cdot L^{-1}$ ATP. The incubation was continued for another 1 min, and then the reduction of ferricyanide (0.1 $mmol \cdot L^{-1}$) was initiated by the addition of 0.2 $mmol \cdot L^{-1}$ of NADH. The light scattering changes of the vesicles in the media containing both Cd^{2+} (0.1 $mmol \cdot L^{-1}$) and Mg^{2+} (2.5 $mmol \cdot L^{-1}$) but without and with ATP were shown in traces A and B, respectively. Trace C shows the effect of including sodium vanadate (0.2 $mmol \cdot L^{-1}$) in the medium containing ATP (1.0 $mmol \cdot L^{-1}$), Cd^{2+} (0.1 $mmol \cdot L^{-1}$) and Mg^{2+} (2.5 $mmol \cdot L^{-1}$). The effects of omitting Cd^{2+} from the Mg^{2+}-containing medium on the scattering of the vesicles, with or without ATP, were shown as trace D. The effects of 10 μg each of nigericin and valinomycin in the medium used for A are shown as traces E and F, respectively.

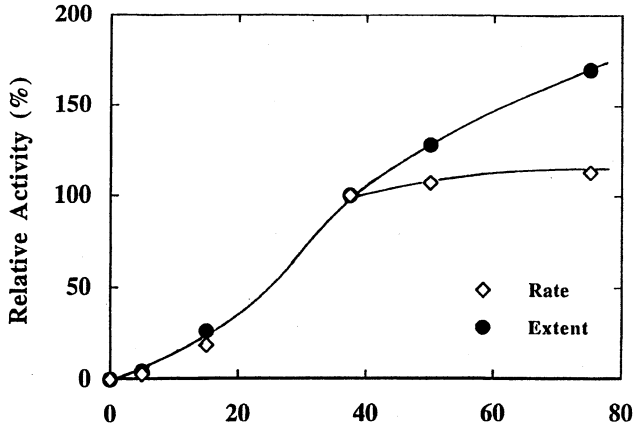

Fig. 2: Dependence of the Light Scattering Change on the Concentration of Cd^{2+}. The plasma membrane vesicles (86 μg) were incubated in the medium containing different concentration of $CdCl_2$ as described in Fig. 1 trace A except with the omission of Mg^{2+}. The maximum rate of light scattering change and the extent of light scattering increase were measured. The relative activities were calculated by assigning the rate and the change obtained with Cd^{2+} concentration as 37.5 $\mu mol \cdot L^{-1}$ as 100 %.

Others

Protein content was determined by a modified Lowry procedure after precipitation by TCA in the presence of deoxycholate (Bensadoun and Weinstein, 1976). Antimycin A, ATP (Na-salt), DTT, EDTA, nigericin, sucrose, valinomycin and all buffers were obtained from Sigma Co. Other chemicals used were of analytical grades.

Results

H^+-ATPase and NADH-linked electron transfer of the plasma membrane

As described in our previous reports, the corn root plasma membrane vesicles exhibited activities of vanadate-sensitive H^+-ATPase (Hsu et al., 1989) and NADH-linked electron transfer (Tu et al., 1996). However, measurements of one activity with the other also activated has never been attempted. To examine whether the activities of the H^+-ATPase may be affected by the activation of NADH-linked electron transfer, experiments shown in Tables 1 and 2 were performed. We have shown that the plasma membrane electron transfer from NADH to ferricyanide remains relatively constant between pH 6 to 8 (Tu et al., 1993). On the other hand, the vanadate-sensitive H^+-ATPase exhibits a distinctive maximum at pH ~6.5 (Hsu et al., 1989). Thus, pH 6.5 was chosen for the experiments. The parameters of proton pumping (Table 1) and the rate of ATP hydrolysis (Table 2) were determined in solutions including none of, part of, and all of the reagents for the complete redox reaction. As shown, the presence of $Fe(CN)_6^{3-}$ caused a noticeable increase in the rate of ATP hydrolysis. However, further addition of NADH to activate the electron transfer to ferricyanide did not greatly alter the ATP hydrolysis rate. While the reasons for ferricyanide stimulation were unknown, the occurrence of electron transfer from NADH to ferricyanide did not further affect the activities of the H^+-ATPase. The effect of initiating the H^+-ATPase activities to the rate of electron transfer was also investigated. As shown in Table 3, the reduction of ferricyanide by NADH in the plasma membrane vesicles was not significantly affected by the activation of ATPase activity. The results, thus, indicated a lack of direct interaction between the electron transfer mechanism and the H^+-ATPase.

Table 1: Effects of the Electron Transfer on H^+-pumping of the ATPase[1].

Experiment	Relative R_0 (%)	Relative k_1 (%)
Control	100	100
+$K_3Fe(CN)_6$	101	107
+NADH	90	98
+$K_3Fe(CN)_6$+NADH	88	97

[1] The measurements of the proton pumping associated with the plasma membrane H^+-ATPase were performed as described in Material and Methods. The membrane vesicles (95 μg of protein) were incubated in the assay medium for 10 min at 22 °C before the addition of redox reagents. ($K_3Fe(CN)_6$, 0.1 mmol · L^{-1}; NADH, 0.2 mmol · L^{-1}). The ATPase-linked activites were then initiated 30 s later by the addition of ATP. The R_0 (initial proton pumping rate) and k_1 (proton leakage with ATPase activity on) of control samples (without the presence of redox reagents) were 0.2680 ΔA min^{-1} mg^{-1} and 0.307 min^{-1}, respectively. The data represented the average of three independent experiments with relative errors ± 10%.

Table 2: Effects of the Electron Transfer on the ATPase Hydrolysis[1].

Experiment	Relative Enzyme Activity (%)
Control	100
+$K_3Fe(CN)_6$	123
+NADH	101
+$K_3Fe(CN)_6$+NADH	130

[1] The experimental procedure and conditions were the same as described in Table 1 except the hydrolysis of ATP as measured by P_i release, was followed. The initital ATP hydrolysis rate for control samples was 484 nmol P_i min^{-1} (mg protein)$^{-1}$. The data represented the average of three independent experiments with errors ± 10%.

Table 3: Effects of Activating the ATPase Activity on the Electron Transfer Rate[1].

Conditions	Rate (μmol e^- transferred min^{-1} mg^{-1})
Control (no Mg-ATP)	2.53, 2.98 (2.76)
H^+-ATPase Activated (+ Mg-ATP)	2.62, 2.74 (2.68)

[1] The measurement of the reduction of ferricyanide was followed as described in text. To test the effects of ATPase activation, 1.0 mmol · L^{-1} ATP was added 2 min prior to the addition of 0.2 mmol · L^{-1} NADH at pH 7.0. The average rates of two independent experiments were listed in parentheses.

Effects of ATPase on electron transfer-induced Cd^{2+} movement

We have shown (Tu et al., 1996) that in Cd^{2+}-containing solution, NADH-linked electron transfer induces a light scattering increase of the plasma membrane vesicles and a concomitant decrease of free Cd^{2+} in solution as illustrated by trace A in Fig. 1. We thus, suggested that the light scattering increase of the membrane vesicles induced by the electron transfer was related to an uptake of Cd^{2+} from the medium. Whether this uptake, measured by the increase in light scattering, could be affected by the activation of the H^+-ATPase was the objective of following experiments.

Because the activation of the ATPase required the presence of $Mg^{2+}$and ATP, we first tested the influence of Mg^{2+} on the light scattering increase. A typical time course of Cd^{2+}-related light scattering increase powered by the NADH-linked electron transfer at pH 6.5, is shown as the trace A in Fig. 1. As shown, the electron transfer process did not induce a light scattering increase in the medium containing Mg^{2+} but without Cd^{2+}. Furthermore, Mg^{2+} also did not have any significant effects on the light scattering increase associated with Cd^{2+} (traces A and D). Thus, Mg^{2+} appeared to have no effect on the electron transfer induced Cd^{2+} movement in

different membrane sites. Unlike the well-studied NADH-linked respiratory electron transfer chain in the inner membrane of mitochondria, that supports a vectorial proton pumping through redox-loop type of mechanisms (Mitchell, 1975), the less well-characterized NADH-linked process in plant plasma membrane vesicles has yet to show direct evidence for inducing electron transfer-linked proton pumping activity (Rubinstein and Luster, 1993; Klobus and Buczek, 1995). The physiological roles of NADH-linked electron transfer in the PM are still obscure. Proposed involvements include the reduction of Fe^{3+} to Fe^{2+} to facilitate iron uptake (Møller and Crane, 1990), hormonal regulation of cell growth, and maintenance of the -SH groups of membrane proteins in a reduced state (Crane et al., 1985; Møller and Crane, 1990). In our previous studies (Tu et al., 1996) we demonstrated that the electron transfer induced a Cd^{2+} uptake-related light scattering increase of corn root plasma membrane vesicles with inside-out orientation. A similar light scattering increase was also observed in medium containing Zn^{2+} or Co^{2+} but not Ca^{2+}. The results suggest that the NADH-linked electron transfer may induce extrusion of certain divalent cations from intact plant root cells.

Since both the ATPase and the NADH-linked electron transfer may be important in powering plasma membrane ion transport, it is of interest to examine whether the two energy-yielding reactions may regulate each other. In present work, we found no evidence for any direct interactions between the H^+-ATPase and the electron transfer process. However, we did find that activation of the ATPase activity reduced the rate of Cd^{2+} movement induced by the NADH-linked electron transfer.

Materials and Methods

Isolation of the plasma membrane

The isolation of microsomal fractions from corn roots followed the procedure described by Brauer et al. (1988). Briefly, corn (*Zea mays* L. Cv. WF7551, Custom Farm Seeds, Co) seeds were germinated on filter paper moistened with 0.1 mmol · L^{-1} $CaCl_2$ for 3 d at 28 °C. Approximately 60 g of excised roots were homogenized with a mortar and pestle at 4 °C in the isolation medium containing 0.3 mmol · L^{-1} sucrose, 5 mmol · L^{-1} ethyleneglycol-bis(β-aminoethylether)-N,N′-tetraacetic acid (EGTA), 5 mmol · L^{-1} β-mercaptoethanol, 5 mmol · L^{-1} dithiothreitol (DTT), and 0.1 mmol · L^{-1} Hepes (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), pH 7.7 (adjusted at 4 °C). After being filtered through four layers of cheesecloth, the homogenate was subjected to differential centrifugation, 6,000 g_n for 20 min and 90,000 g_n for 40 min, to obtain a microsomal pellet. The microsomal pellet was suspended in isolation medium and centrifuged again at 90,000 g_n for 40 min. The washed microsomes were suspended in the isolation medium (2 mg protein mL^{-1}) and layered over a discontinuous density gradient consisting of 34 and 42 % (w/w) sucrose buffered with 5 mmol · L^{-1} Hepes, pH 7.5. After being centrifuged at 100,000 g_n for 150 min, the plasma membranes collecting between the 34 and 42 % sucrose layers were removed as described by Hsu et al. (1989), diluted 3- to 4-fold with 5 mmol · L^{-1} Hepes (pH 7.5) and then centrifuged at 100,000 g_n for 90 min. The final pellet was resuspended in 20 mmol · L^{-1} Hepes (pH 7.5), 0.2 mmol · L^{-1} sucrose, and 10 % (w/v) glycerol at 5 mg protein mL^{-1}.

Measurement of the H^+-ATPase activities

In general, about 100 µg of purified plasma membrane vesicles, were diluted with 2.0 mL of assay medium containing 20 mmol · L^{-1} Mes titrated to different pHs, 2.5 mmol · L^{-1} $MgSO_4$, 0.1 mmol · L^{-1} molybdate, 100 mmol · L^{-1} KCl, 50 mmol · L^{-1} KNO_3, 5 mmol · L^{-1} glucose, 17.5 µmol · L^{-1} acridine orange (AO), and other additions as specified in text and were incubated at 22 °C for 10 min. The ATPase activities were then initiated by the addition of 20 µL of 0.1 mol · L^{-1} ATP titrated to 6.45 with BTP. Specific procedures for ATP hydrolysis and proton pumping activities are as follows:

a). *ATP hydrolysis:* The ATP hydrolysis catalyzed by the plasma membrane was terminated by the addition of 1 mL of ice-cold 5 % TCA 10 min after the initiation of the reaction. The amount of phosphate released was determined by the malachite green-molybdate assay as previously described (Tu et al., 1987). At least 70 % of the total ATP hydrolysis was catalyzed by the plasma membrane H^+-ATPase based on the sensitivity to 0.2 mmol · L^{-1} vanadate. The vanadate-sensitive ATP hydrolysis rate (difference between with and without 0.2 mmol · L^{-1} vanadate) remained constant for about 15 to 20 min under the experimental conditions. Thus, the average rate over a time period of 10 min was used to represent the initial hydrolysis rate of the H^+-ATPase. The inclusion of NADH and/or ferricyanide after acid quenching did not have any effect on the determination of phosphate by the present method.

b). *Proton pumping measurement:* ATP-driven H^+-transport was followed by changes in absorbance of AO at 492.5 nm in the same reaction mixture used for ATP hydrolysis measurements. It should be mentioned that the NADH and/or ferricyanide, under the experimental conditions, did not alter the response of AO to pH change. The time courses of the absorbance change were followed by a Beckman DU-70 Spectrophotometer interfaced to a personal computer. The digitized data (sampling time = 1 s) were then analyzed according to a steady-state kinetic model developed in this laboratory (Tu et al., 1987).

Measurements of electron transfer and its related Cd^{2+} movement

Typically plasma membrane vesicles with ~100 µg protein were diluted to 2 mL in the same buffer used for the ATPase measurements except that AO was omitted and 50 or 100 µmol · L^{-1} $K_3Fe(CN)_6$ and various concentrations of different divalent cations as described in legends were added. After acquiring a stable flat baseline, 200 µmol · L^{-1} NADH was added to initiate the reduction of ferricyanide and the light scattering change. The reduction of ferricyanide catalyzed by the membrane was monitored at 22 °C by absorbance decrease at 420 nm. To quantify the reduction, the difference in the extinction coefficients of ferri- and ferrocyanides (−0.93 mmol · L^{-1} cm^{-1}) was used. This difference was not affected by the presence of Mg-ATP under the experimental conditions. The light scattering change of the membrane vesicles in Cd^{2+}-containing medium induced by the electron transfer was monitored spectroscopically at 660 nm at 22 °C.

Based on microscopic measurement and digital imaging analysis, we have shown that the scattering increase observed under the experimental conditions, reflects mainly an osmotic swelling but not aggregation or fusion of the vesicles (Tu et al., 1996). The kinetics of the scattering increase consists of a complex pattern which normally contains an initial lag period followed by a rapid increase to a maximum rate and then a decrease to zero rate, as the swelling ceases. Since the osmotic swelling is more closely related to the movement of solvent rather than to the movement of solutes, the observed scattering change, as described in our previous study (Tu et al., 1996), may only be used as a qualitative/semi-quantitative indication for Cd^{2+} uptake by the vesicles.

A Calcium-Dependent Protein Kinase is Associated with Maize Mesocotyl Plasmodesmata

AVITAL YAHALOM, REVITAL LANDO, AVIVA KATZ, and BERNARD L. EPEL*

Department of Plant Sciences, George S. Wise Faculty of Life Science, Tel Aviv University, Ramat Aviv, Tel Aviv, 69978, Israel

Received July 7, 1997 · Accepted January 30, 1998

Summary

Plasmodesmata (Pd) are trans-wall membrane lined tunnels that regulate cell-to-cell cytoplasmic movement. It has been suggested that Pd conductivity may be regulated by a phosphorylation mechanism. In a maize (*Zea mays* L.) mesocotyl cell wall fraction, a Ca^{2+}-dependent protein kinase (CDPK) is present that phosphorylates approximately 8 of 20 wall-associated proteins. The kinase is membrane-associated and is not extracted by EGTA, NaCl, up to 4 mol/L LiCl, Triton X-100, or Na_2CO_3 (pH 11), but is fully extracted with SDS or 8 mol/L LiCl. Two polypeptides in the cell wall fraction, with apparent molecular masses of 51 and 56 kD, cross-react with an *Arabidopsis* CDPK anti-serum and undergo *in situ* Ca^{2+}-dependent autophosphorylation on nitrocellulose. The molecular masses of the CDPKs extracted by 8 mol/L LiCl from the cell wall fraction are different from those extracted from the cell membrane fraction, suggesting that the wall-associated CDPK is unique to the cell wall fraction. Immuno-fluorescence microscopy with isolated walls localizes CDPK to discrete punctate loci in the cell wall. Isolated Pd challenged with CDPK anti-serum show a pattern of cross-reactivity similar to the cell wall fraction. These data suggest that the cell wall-associated CDPK is a putative plasmodesmal-associated membrane protein and may be involved in regulating Pd conductivity.

Key words: Zea mays, cell wall, calcium-dependent protein kinase, phosphorylation, plasmodesmata.

Abbreviations: CW = cell wall fraction; CDPK = calcium-dependent protein kinase; HM = homogenization media; MF = cell membrane fraction; Pd = plasmodesmata; PMSF = phenyl methyl sulphonyl fluoride; PB = phosphorylation buffer; TEM = transmission electron micrograph.

Introduction

Plasmodesmata (Pd) are plant-specific, trans-wall tunnels that regulate cell-to-cell cytoplasmic movement of molecules. Current models of Pd substructure describe them as complex pores lined with plasma membrane having a central membranous axial component termed the desmotubule or appressed ER (Robards and Lucas, 1990; Ding et al., 1992; Botha et al., 1993). Particles, presumably proteinaceous but with unknown identity or function, are present within the Pd (Ding et al., 1992; Botha et al., 1993). Recent evidence suggests that both environmental and developmental signals can alter the size-exclusion limits of these conducting tunnels, thus governing Pd conductivity (Epel, 1994; McLean et al., 1997). In addition, it appears that Pd also selectively transport proteins and nucleic acids (Lucas et al., 1995; Waigmann and Zambryski, 1995). Although the gating mechanism of Pd remains unresolved, it seems likely to involve phosphorylation processes (Epel et al., 1994; McLean et al., 1997). Second messengers including Ca^{2+} and inositol phosphates, which activate protein kinases, close Pd (Erwee et al., 1983; Tucker, 1988; Tucker, 1990; Tucker and Boss, 1996), while treatments like azide and anaerobic conditions that decrease cellular ATP levels open Pd (Tucker, 1993; Cleland et al., 1994). Based on these findings, it has been suggested that Pd conductivity may be regulated by one or more Pd-associated protein kinases and/or phosphatases (Epel et al., 1994; McLean et al., 1997).

* Correspondence.